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Posted Date: 10 April 2023

doi: 10.20944/preprints202304.0134.v1

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Article

Chemical Profiling of Diffusible and Volatile Secondary Metab-Olites Produced by Beauveria bassiana Using GC-MS Analysis: In Vitro Antimicrobial Activity

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Abstract: The genus *Beauveria* include important entomopathogenic and endophytic fungi, among them, *B. bassiana* is the most studied species. However, there are few knowledge regarding their antimicrobial activity. The current research has been conducted to evaluate *in vitro* antibacterial efficacy of five isolates of *B. bassiana* against *Bacillus cereus, B. megaterium, B. mojavensis, Clavibacter michiganensis* (gram positive bacteria, G+ve), *Xanthomonas campestris, X. vesicatoria, Escherichia coli, Pseudomonas aeruginosa* and *P. fluorescence* (gram negative bacteria, G-ve). In addition, chemical composition of the principal diffusible metabolites and volatile organic compounds (VOCs) of the most efficient studied isolate of *B. bassiana* has been carried out using GC-MS analysis. The obtained results showed that, the isolate UniB2439-3 has promising antibacterial effect against most of studied target bacteria. GC-MS analysis of diffusible metabolites detected the presence of hexanedioic acid, bis(2-ethylhexyl) ester as the main compound in the cell-free culture filtrate. Furthermore, GC-MS analysis of VOCs revealed the presence of ethanol; butanal,2-methyl; 2,4-dimethyl-1-heptene; octane, 4-methyl and β-elemene as the main dominant bioactive compounds. The outgoing results explicated that, the isolates of *B. bassiana* have promising antibacterial activity which could be correlated to their diffusible and VOCs metabolites. Therefore, the selected isolate can be potentially used as a biocontrol agent against several bacteria especially G+ve ones. Taking in consideration that the antibiotics are forbidden in agriculture in many countries worldwide, search for possible natural alternatives as efficient antimicrobial agents are highly interesting.

Keywords: biocontrol; natural products; phytopathogens; microbial metabolites; entomopathogens; endophytic fungi

1. Introduction

Genus *Beauveria* include entomopathogenic and endophytic fungi which are widely spread in different habitats [1–3]. Furthermore, many researchers reported that fungi in the genus *Beauveria* can produce enzymes for biotransformation and biodestructors [4,5]. On the other hand, the genus *Beauveria* is considered also a human pathogen where it causes opportunistic infections especially in patients with weak immune systems [6].

Some species of this genus, such as *B. bassiana* and *B. brongniartii* are able to produce mycoinsecticides [7]. *B. bassiana* is also a beneficial microorganism (BM) and endophytic fungi (EF) in several crops and is commonly known as biological control agent against a variety of agricultural pests [3,8–10]. The application of *B. bassiana* has many advantages such as being an eco-friendly management compared to chemical pesticides where it is harmless to human health [8,10–12]. For decades, several scientists have reported the importance of *B. bassiana* in reducing a range of nuisance

insects, where it can induce direct insect mortality [2,13,14] and can also reach 90% reduction of life-time fecundity [15].

A recent study, conducted by Barra-Bucarei et al. [16] to evaluate the colonization ability of native endophyte of different strains of *B. bassiana* and their antifungal effect against *Botrytis cinerea* in tomato and chili pepper concluded that all studied strains had significant *in vitro* antagonism against *B. cinerea.* The same study reported that, the native strains of *B. bassiana* were able to colonize tomato and chili pepper tissues and provided important levels of antagonism [16].

Another research conducted by Sinno et al [10] has evaluated different isolates of *B. bassiana* as plant-growth promoting (PGP) and protective agent for tomato plants against *B. cinerea*, *Alternaria alternata* and the pest aphis, *Macrosiphum euphorbiae*. The results showed that some studied isolates were able to control the two phytopathogens, whereas one isolate was also able to promote plant growth [10]. The antibacterial activity of crude ethyl acetate extract of *B. bassiana* against some aerobic pathogenic bacteria been tested by Parine et al [17]. The results explicated that the extract of *B. bassiana* possess a strong inhibiting activity against many of tested species especially *Bacillus megaterium*, *B. subtilis*, *B. sphaericus* and *Escherichia coli* [17]. Whereas, it showed a moderate effect against *Micrococcus luteus*, *Pseudomonas aeruginosa* and low effect against *Streptococcus pyogenes* and *Chromobacterium violaceum* [17]. In another study, the application of conidia of *B. bassiana* has protected tomato seedlings from damping-off disease caused by the soil-borne pathogen *Rhizoctonia solani* [18].

Recently, there is a huge interest for discovering natural substances based plant or microbe origins having herbicidal and/or pesticide effect [19,20]. However, the new discovered natural substances should be evaluated for safety to avoid any possible negative health impact [21,22]. In addition, the discovery of possible natural alternatives for reducing the excessive use of synthetic chemicals, decreasing the environmental hazards and avoiding the appearance of new resistance microbial strains to common microbicide compounds should be highly considered [18,23].

There are few information regarding the bioactive metabolites produced by *B. bassiana* either diffusible or volatiles and also their mechanism of action in the antimicrobial activity and plant growth promotion effect. A recent study conducted by Wang et al [24] reported that *B. bassiana* produces a variety of toxins such as beauvericin, bassianin, bassianolide, beauverolides, tenellin, oosporein and oxalic acid, which give the ability to *B. bassiana* to colonize, parasitize and kill the host tissues. Therefore, the precise chemical characterization and determination of the main bioactive single substances of *B. bassiana* will certainly aid in understanding its biological importance. In addition, the details of chemical constituents of *B. bassiana* will undoubtedly serve for various applications such as control plant diseases, taking in consideration the heavy reliance on chemicals that are extremely harmful to environment as well as plants, animals and human health. Furthermore, there are many studies have been carried out regarding the insecticidal effect of *B. bassiana*, but there are few studies undersigned their antifungal or antibacterial effect.

The main objective of this research is to study the chemical composition of the principal diffusible metabolites and volatile organic compounds (VOCs) of *B. bassiana* and shed light on the possible transformation and/or fragmentations of its different chemical derivatives. Hence, the chemical composition of *B. bassiana* metabolites will aid in the detection and differentiation of this species from others. The full identification of each single component can help in its utilization in industrial, agricultural and pharmaceutical field. In addition, in this research we will expand the possible benefits of *B. bassiana* against new non-reported target phytopathogens. In particular, the aims of the current research were to i) evaluate the antagonistic activity of five isolates of *B. bassiana* against some phytopathgenic bacteria; ii) investigate *in vitro* antimicrobial activity of secondary metabolites extracted from the most efficient isolate; iii) chemically characterize the diffusible secondary metabolites (DSMs) and volatile organic compounds (VOCs) obtained from the most efficient isolate using GC-MS analysis.

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2. Results

2.1. Molecular identification of the studied isolates of Beauveria

The PCR amplification with Bt2a/Bt2b produced, for each gDNA extracted from the above five isolates (UniB2439-1; UniB2439-2, UniB2439-3, UniB2439-4, UniB2439-5), amplicons with molecular weight about 330 bp. No amplification was observed in case of the negative control. The amplicons were directly sequenced (BMR Genomics, Padova, Italy) and the obtained sequences were compared with those available in GenBank nucleotide archive (AB829899; AB829898 and CP045886.1) using Basic Local Alignment Search Tool software BLAST (Bethesda, Rockville Pike, MD, USA) [25]. The sequences analysis showed high similarities percentages with the sequences of *B. bassiana.* The five obtained sequences were deposited in the NCBI GeneBank with accession numbers FR989662 to FR989666.

2.2. Antagonistic activity of B. bassiana isolates

The preliminary results showed that, all tested isolates of *B. bassiana*, had antagonistic effect against most of tested bacterial strains. Particularly, the efficient isolate was UniB2439-3, where it showed the highest significant effect against *B. cereus*, *B. mojavensis* and *C. michiganensis*, moderate effect against *B. megaterium* and *X. vesicatoria*, low effect against *X. campestris* and *P. fluorescens*, whereas no activity was observed in case *P. aeruginosa* (Figure 1). Therefore, the isolate UniB2439-3 was selected for further biological and chromatographic analyses.

2.3. Antimicrobial activity of secondary metabolites

The obtained results of the extracted metabolites from the selected isolate of *B. bassiana* UniB2439-3 showed that, the *Exo*-ME was able to inhibit the growth of most tested bacterial strains higher than the *Endo*-ME (Table 1). In particular, *Exo*-ME showed the highest significant activity against *X. vesicatoria*, *B. mojavensis* and *C. michiganensis* (Table 1). In addition, both extracts showed equal activity against *X. campestris*. Whereas, only *Endo*-ME showed antibacterial activity against *P. aeruginosa* (Table 1). On the other hand, *Endo*-ME was not active against *X. vesicatoria*, *B. cereus*, *B. mojavensis* and *C. michiganensis* (Table 1). Although both extracts were lower efficient than the control (Tetracycline), but they can be considered promising and optimistic antimicrobial agents, being natural biopesticides can be potential alternatives to chemical and synthetic antibiotics.

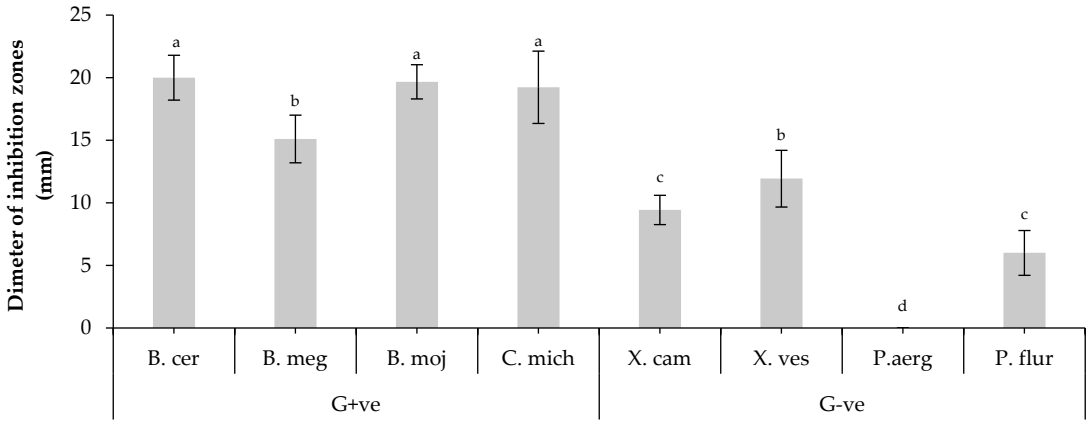

Figure 1. Antagonistic activity of *B. bassiana* UniB2439-3.

Bars with different letters are significantly different according to one-way ANOVA combined with *Tukey* B post hoc multiple comparison test at $P < 0.05$ using SPSS statistical analysis software. Data for each bar are expressed as the mean of three replicates ± SDs.

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Table 1. Antibacterial activity of diffusible metabolites from *B. bassiana* UniB2439-3.

	Tested bacteria	Diameter of inhibition zones (mm)		
		***Exo*-ME** 16 mg/mL	***Endo*-ME** 20 mg/mL	**Tetracycline** 1600 µg/mL
G+ve	*B. cereus*	8,5±1,0ab	0,0±0,0c	20.8±1.1b
	B. megaterium	10,0±1,9ab	4,0±1,7b	25.9±2.3ab
	B. mojavensis	14,0±2,6a	0,0±0,0c	18.4±1.4b
	C. michiganensis	12,5±2,2a	0,0±0,0c	39.5±2.5a
G-ve	*X. campestris*	9,5±2,5ab	9,0±1,9a	23.5±1.7ab
	X. vesicatoria	14,0±0,9a	0,0±0,0c	19.5±0.9b
	P. aeruginosa	0,0±0,0c	6,5±2,8ab	10.6±0.7c
	P. fluorescens	6,5±1,5b	4,5±1,7b	12.3±0.9c

Values followed by different letters in each column for each tested extract against all tested bacteria are significantly different at $P < 0.05$ according to one-way ANOVA combined with *Tukey* B post hoc test by using SPPS program. Data are expressed as the mean of inhibition zone diameter (mm) for three replicates ± SDs compared to controls ±SDs.

2.4. GC-MS analysis of secondary metabolites

2.4.1. Diffusible metabolites

The GC-MS analysis of diffusible metabolites detected the presence of hexanedioic acid, bis(2-ethylhexyl) ester as the main diffusible compound in the cell-free culture filtrate of *B. bassiana* UniB2439-3 with molecular weight 370 and relative area 100% (Table 2).

Table 2. GC-MS analysis of diffusible metabolites extracted from *B. bassiana* UniB2439-3.

RT[a] (min)	Area (%)	Name	M.Wt[b] (g/mol)	Formula	CAS[c]	Quality (%)
14.819	100.0	Hexanedioic acid, bis(2-ethylhexyl) ester	370	$C_{22}H_{42}O_4$	000103-23-1	91

Where: [a.] RT: retention time; [b.] M.Wt: molecular weight, and [c.] CAS: registry number of chemical compound.

2.4.2. Volatile metabolites (VOCs)

GC-MS analysis of the VOCs produced by *B. bassiana* UniB2439-3 showed that the dominant principal compounds, listed in Table (3), are: i) ethanol, ii) butanal,2-methyl, iii) 2,4-dimethyl-1-heptene, iv) octane, 4-methyl and v) β-elemene among the total 34 detected volatile compounds (Table S1). In particular, the most abundant constituents were ethanol and β-elemene with relative areas 4.69 and 6.98 %, respectively.

Table 3. GC-MS analysis of VOCs extracted from *B. bassiana* UniB2439-3.

RT (min)	Area (%)	Name	M.Wt (g/mol)	Formula	CAS	Probability of identification (%)
1.576	4.69	Ethanol	46,07	C_2H_5OH	000064-17-5	90
2.834	0.44	Butanal, 2-methyl	86	$C_5H_{10}O$	000096-17-3	90
5.372	00.63	2,4-Dimethyl-1-heptene	126.24	C_9H_{18}	019549-87-2	90
5.660	1.99	Octane, 4-methyl	128.25	C_9H_{20}	002216-34-4	93
10.459	6.98	β-elemene[a]	204.35	$C_{15}H_{24}$	000515-13-9	96

[a.] β-elemene: Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)].

 doi:10.20944/preprints202304.0134.v1

3. Discussion

Several research have been conducted recently to overcome the multi-drug resistant (MDR) microorganisms to different antibiotics and chemotherapeutic agents [26]. Hence, the search for new active and natural agents get a great interest, particularly, for human health and environmental protection [27]. *Beauveria*, one of the most studied genus among entomopathogenic fungi, has various biological applications as growth promoting agent or insecticides [9,10,28,29]. The capacity of *B. bassiana* to produce several diffusible or volatiles bioactive metabolites with promising antimicrobial properties is in agreement with the previous bibliographic research who investigate the antagonistic effect against several phytopathogens [9,17,30]. In fact, the bibliographic research revealed that the genus *Beauveria* produced some interesting metabolites such as oosporein, beauvericin, bassianolide, bassianin, beauveriolide, bassiacridin and cyclosporine which having notable insecticide and antimicrobial actions [31,32]. On the other hand, some of the produced metabolites from *B. bassiana* could contain interesting cell-wall hydrolytic enzymes which are able to degrade microbial cell wall and hence inhibition the growth of several phytopathogens [33].

On the other hand, Barra-Bucarei et al [16] studied the antifungal activity of ten native strains of *B. bassiana*, endophyte for tomato and chili pepper and observed that the majority of studied native strains were able to colonize tomato and chili pepper tissues and showed promising antagonistic effect against *B. cinerea.*

The obtained results of GC-MS analysis of diffusible metabolites extracted from *B. bassiana* UniB2439-3 revealed the presence of hexanedioic acid, bis(2-ethylhexyl) ester which has been identified in different bacteria such as *Sterptomyces* spp., showed promising antimicrobial activity against several human and/or food pathogenic bacteria such as *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* and also against the phytopathogenic fungi, *Fusarium* sp. [34].

Furthermore, Hernández-Ceja et al [35] reported that the extract from *Adenophyllum porophyllum* has strongly inhibited the mycelial growth of the pathogenic fungi *Pestalotiopsis clavispora*, *Colletotrichum gloeosporioides* and *Lasiodiplodia pseudotheobromae,* responsible for the dieback disease of blueberry (*Vaccinium* spp). The same authors have analyzed the chemical constituents extracted from *A. porophyllum* by GC-MS and concluded that hexanedioic acid, bis(2-ethylhexyl) ester was the major compound present in the *A. porophyllum* extract [35]. In addition, Bai et al [36] analyzed the chemical constituents of the supercritical fluid extract from roots of *Stellera chamaejasme* and found that the hexanedioic acid, bis (2-ethylhexyl) ester, among 12 dominant single compounds detected, showed antifungal activity against *Monilinia fructicola*.

Regarding the VOCs produced by *B. bassiana* UniB2439-3, the obtained results are in agree with many research which reported that oosporein, beauvericin, bassianolide, bassianin, beauveriolide, bassiacridin and cyclosporine are the most abundant metabolites produced by *B. bassiana* [37–39]. In particular, in our study, it was observed that the beauvericin was fragmented into diethyl phthalate with 90%, the major dominant constituent, carbon dioxide and nitrous oxide (Table S1). Whereas, bassianolide was converted to butanal, 3-methyl with 81% or butanol, 3-methyl with 83% (Table S1). Regarding bassianin, GC-MS analysis showed that this compound was converted into 2,4-Dimethyl-1-heptene with 90% (Table S1). Whereas, beauveriolide was fragmented into butanal, 3-methyl with 81%, carbon dioxide and nitrous oxide (Table S1). Regarding cyclosporine, results demonstrated that this compound was fragmented into butanal, 2-methyl- (90%), butanal, 3-methyl- (81%), 1-butanol, 3-methyl- (83%), carbon dioxide and nitrous oxide (Table S1).

Several studies revealed that beauvericin and oosporein evidenced remarkable antibiotic and antifungal properties [40,41] which are probably involved in the microbial growth inhibition observed in the bioassay presented in this study Furthermore, Wang and Xu [42] reported that beauvericin was one of the active constituents of *B. bassiana* and confirmed to have antimicrobial activity and anti-tumor effect especially against human leukemia. In another study, conducted by Manning and Wyatt [43], the results demonstrated that oosporein, extracted from the broth cultures of *Beauveria* and *Chaetomium*, has been identified as a toxic substance for plants and poultry.

 doi:10.20944/preprints202304.0134.v1

Regarding the β-elemene, our obtained results detected the presence of an important sesquiterpene compound identified as β-elemene (cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl), 1S-(1.alpha.,2.beta.,4.beta.), among the detected VOCs substances from the studied *Beauveria* isolate. This compound (β-elemene) was identified for the first time in 1994 in the dry rhizome extract from *Curcuma phaeocaulis*, *C. kwangsinensis* and *C. wenyujing* [32]. In addition, β-elemene is also one of the common constituents of several aromatic essential oils extracted from *Proteus vulgaris* [44]. β-elemene was also found in wild hops from Lithuania at levels up to 14% [45] and in notable amounts in the medical cannabis cultivar 'bedropuur' [46]. The same compound has notable antimicrobial activity against different pathogens including *Mycobacterium tuberculosis* as reported by Sieniawska et al. [47].

Generally, the mechanism of the antimicrobial activity of several terpenes is highly related to their lipophilic property which enable them to dissolve in the phospholipid layers of microbial cell membrane [48]. Particularly, natural sesquiterpenes such as β-elemene originated from plant and microorganisms showed promising antimicrobial activity [49,50]. A recent study conducted by Monga and Sharma [51] reported that β-elemene and R-limonene playing an essential role in degrading the microbial cell wall altering the expressions of *dprE1* and *clgR* genes, responsible for the cell wall synthesis and cell membrane preservation, respectively.

On the other hand, some recent studies reported the promising cytotoxic effect of β-elemene which can inhibit cell proliferation, arrest cell cycle and induce cell apoptosis or autophagy [52]. β-elemene is one of the most promising inhibitors of glycolysis rate-limiting enzyme especially (PKM2) through the interfering with tumor glycolysis which consider one of the most important recent strategy for treating tumors [53,54]. In fact, several research reported that the inhibition of tumor growth and proliferation can be achieved by down-regulating expression of PKM2 enzyme [55]. In addition, Pan et al. [56] pointed to the role of β-elemene in inhibiting the breast cancer cell migration by conversion the dimer and tetramer forms of PKM2 and inhibit the aerobic glycolysis and reduce the utilization of glucose and the production of lactic acid for tumor cell growth.

4. Materials and Methods

4.1. Isolation, culturing and identification

Five strains of *Beauveria bassiana* (UniB2439-1; UniB2439-2, UniB2439-3, UniB2439-4, UniB2439-5), isolated from different soil and vegetal samples, were identified based on morphological features and molecular basis. For molecular identification, the total gDNA was extracted and amplified using the universal primer Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC) [57]. The obtained amplicons were sequenced and then analyzed using Basic Local Alignment Search Tool software (BLAST-USA). The studied isolates were maintained as lyophils at 4°C in the fungal collection of School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), University of Basilicata, Potenza, Italy. The subcultures were carried out on Sabouraud Dextrose Agar plus 1% Yeast-extract (SDAY) nutrient media [5] and incubated at 22±2°C for 96 hrs [58].

4.2. Antagonistic activity

The antagonistic activity of the five studied isolates of *B. bassiana* was evaluated against some pathogenic bacteria. All tested isolates were obtained from the pure cultures conserved in the collection of SAFE and previously identified based on morphological and molecular methods. The tested bacteria are listed in Table (4).

The antibacterial assay was carried out as described by Elshafie et al [59]. About 0.5 cm fungal disc from the fresh PDA culture (96 hrs) of each studied isolates of *B. bassiana* were deposited in the center of KB Petri dish and incubated for 16 hrs at 22±2°C. Successively, a suspension of soft-agar (0.7%) of each tested bacteria at 10^8 CFU/ml was sprayed over the plates using Eco-Spray Ecological Aerosol (Seidden Identificación, Madrid – Spain). All plates were incubated at 30°C for 24 hrs. Two KB plates inoculated only with each tested bacteria were used as negative control. The experiment

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was run in triplicate and the diameter of inhibition zone was measured with a caliber and recorded as the mean ± SD (n = 3). The antagonistic bacterial activity percentage (ABP %) was calculated using the Equation (1):

$$ABP\ (\%) = D.iz/D.ct \times 100; \quad (1)$$

Where ABP: is antagonistic bacterial activity; D.iz: is the diameter of inhibition zones in cm; D.ct: is the diameter of control plates in cm.

Table 4. The tested bacterial strains in the current study.

Bacteria name	Author	Collection number	Gram type
B. cereus	Frankland & Frankland	UniB12421	G+ve
B. megaterium	de Bary	UniB12421	
B. mojavensis	Roberts	UniB10718	
C. michiganensis	(Smith) Davis	UniB3718	
X. campestris	(Pammel) Dowson	UniB7718	G-ve
X. vesicatoria	(Doidge) Dye	UniB8718	
P. aeruginosa	(Schröter) Migula	UniB02421	
P. fluorescens	(Flügge) Migula	UniB05421	

All tested bacteria, with a collection number for each strain, are conserved in the collection of SAFE, University of Basilicata, Potenza, Italy.

4.3. Extraction of secondary metabolites

On the base of the preliminary antagonistic assay, the most efficient isolate (UniB2439-3) of *B. bassiana* was selected for the successive studies. For this scope, 2 mL of the fungal suspension (10^6 spore/mL) of the above isolate, was used for inoculating 500 mL SDY broth nutrient media and then incubated for 7 days at 25°C in agitation (180 rpm). Both, Extracellular (*Exo*-ME) and Endocellular (*Endo*-ME) metabolites have been extracted from the broth culture after the incubation period.

For *Endo*-ME, the incubated broth culture was centrifuged at 40,000 *g* for 15 min and the pellet (2 g) was collected, resuspended in 50 mL of Limonene (CAS 138-86-3- Aldrich, Steinheim, Germany), shaked for 2 hrs and the solvent was evaporated after that using the Rotary-evaporator (Heidolph WB2000, Schwabach, Germany). The residue was resuspended in 2 ml of sterile distilled water (SDW), extracted following Solid Phase Extraction (SPE) by using C-18 column (Thermo Scientific, Rockwood, USA) and recovered using 1 mL methanol to reach the final original concentration of (20 mg/mL) [23].

For *Exo*-ME, the supernatant (250 mL), obtained from the above centrifugation step, was filtered using 0.22 µm (Syringe filter - hydrophilic, Minisart, Goettingen, Germany) and extracted using a separator funnel containing 250 mL ethyl acetate/ethanol (70:30; *v*/*v*) and shacked for 15 min. The organic phase was filtered through a filter paper (Whatman, Ø. 25 mm, Merck KGaA, Darmstadt, Germany) and evaporated using the Rotary-evaporator. The dry residue (50 mg) was resuspended in 2 mL SDW, extracted through SPE using C-18 column and recovered using 1 mL methanol to reach the final original concentration of (16 mg/mL) [23].

4.4. Antibacterial activity of extracted secondary metabolites

The antibacterial activity of both metabolites extracts was carried out against the same pathogenic bacteria used for the initial antagonistic assay, listed in Table (1).

Disc diffusion assay. The antibacterial test of both metabolites extracts produced by the most bioactive isolate UniB2439-3 was carried out following the disc diffusion method as described by Elshafie el al [60] and Sofo et al. [61]. A bacterial suspension of each tested bacteria was prepared in sterile distilled water adjusted at 10^6 CFU/mL (OD ≈ 0.2 nm) using UV-Spectrophotometer (Amersham, Ultraspec 1100 pro/500 pro, UK). Four mL of bacterial suspension mixed with soft agar 0.7 % (9:1; *v*/*v*) was poured over each KB plate (9 Φ cm). Blank discs of 6 mm (OXOID, Milan-Italy) were then placed over the plates and 15 µL from each tested metabolites extract (*Exo*-ME 16 mg/mL

and *Endo*-ME 20 mg/mL) was carefully applied over discs. Tetracycline (1600 µg/mL) was used as a positive control. The experiment was performed in triplicates and the antibacterial activity was estimated by measuring the diameter of inhibition zone in mm ± SDs compared to the positive control ones.

4.5. Chemical characterization of secondary metabolites

4.5.1. GC-MS analysis of Exo-diffusible metabolites

On the base of the results from the antibacterial assay of the extracted metabolites, the most bioactive extract (*Exo*-ME) was selected for the successive chemical characterization. A qualitative analysis of the SPE methanol extract of the selected *Beaveria* isolate was carried out using GC-MS (Agilent HP6890) equipped with a Phenomenex Zebron ZB-5 MS capillary column 30 m x 0.25 mm ID x 0.25 µm film thickness (Conquer Scientific, Poway, California, USA). A HP 5973 mass selective (mass range: 15-800 mAU; scan rate: 1.9 scan/s; EM voltage: 1435) was used as detector, whereas helium at 0.8 ml/min was used as carrier gas. The injection port, equipped with a glass insert (internal diameter 0.75 mm) was splitted at 250°C and the desorption time of 1.0 min was used. Detector was maintained at 230°C. Oven was maintained at 80°C for 3 min, then the temperature was increased until 250°C (20°C/min) for 10 min. All the analyses were performed in triplicates. The chromatogram obtained from the total ion current were integrated without any correction for coelutions and the results were expressed as percent of the total area of peaks. All peaks were identified from their mass spectra by comparison with those present in Wiley 6N and NIST11 libraries [59,62].

4.5.2. GC-MS of volatile organic compounds

The fresh culture (96 hrs) of the selected *Beaveria* isolate was inoculated in glass tube of 10 ml PDA nutrient media and incubated at 22°C for 5 days under darkness for collecting the volatile organic compounds (VOCs) as described by Elshafie et al [63]. The eventually produced VOCs have been analyzed qualitatively using Solid Phase Micro Extraction method (SPME) as discussed below.

The SPME fiber coated with 100 µm of non-grafted poly (dimethylsiloxane) phase (Supelco 57300-U, mounted on a Supelco 57,330 support- Merck KGaA, Darmstadt, Germania) was conditioned for 1 h at 250°C in a stream of helium. A blank run was performed after each analysis in order to confirm that no residual compounds were polluting the fiber or the column. The fiber was later introduced into the injection port of a HP6890 plus gas chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness). A HP 5973 mass selective (mass range: 15-800 mAU; scan rate: 1.9 scan/s; EM voltage: 1435) was used as detector, whereas helium at 0.8 mL/min was used as carrier gas. The injection port, equipped with a glass insert (internal diameter 0.75 mm) was splitted at 250°C. The desorption time of 1.0 min was used. Detector was maintained at 230°C. Oven was maintained at 80°C for 3 min, then the temperature was increased until 250°C (20°C/min) for 10 min. All the analyses were performed in triplicate. The chromatograms obtained from the total ion current were integrated without any correction for coelutions and the results were expressed as percent of the total area of peaks. All peaks were identified from their mass spectra by comparison with those present in Wiley 6N and NIST11 libraries [62,63].

5. Conclusions

B. bassiana, apart from being a notable entomopathogenic fungi or biocontrol agent against some phytopathogenic fungi, itself or its bioactive metabolites could be also used efficiently to control several bacteria in agronomic field where it is forbidden to utilize antibiotic especially in organic farming. In addition, *B. bassiana* could be also useful biocontrol agent against MDR microorganisms to different antibiotics which are considered a dominant medical problem worldwide. The obtained results from the current research concluded that, *B. bassiana* UniB2439-3, was able to produce some interesting bioactive secondary metabolites either diffusible as hexanedioic acid, bis(2-ethylhexyl) ester or VOCs as ethanol; butanal,2-methyl; 2,4-dimethyl-1-heptene; octane, 4-methyl and β-elemene.

The ability of *B. bassiana* to produce the above-mentioned metabolites can underling its antagonistic activity against several phytopathogens as reported previously in the bibliographic research. Future studies remain necessary for evaluation the *in vivo* antimicrobial activity of each single identified bioactive metabolites from *B. bassiana* against some common human and phytopathogens.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Chromatogram of the purified diffusible metabolites of *B. bassiana* UniB2439-3; Figure S2: Mass spectra of Hexanedioic acid, bis(2-ethylhexyl) ester; Figure S3: Chromatogram of VOCs extracted from *B. bassiana* UniB2439-3; Figure S4: Mass spectra of ethanol; Figure S5: Mass spectra of Butanal, 2-methyl; Figure S6: Mass spectra of 2,4-Dimethyl-1-heptene; Figure S7: Mass spectra of Octane, 4-methyl; Figure S8: Mass spectra of β-elemene. Table S1: The whole list of GC-MS analysis of VOCs extracted from *B. bassiana* UniB2439-3.

Author Contributions: Conceptualization, Ippolito Camele and Hazem Elshafie; Data curation, Sadeek Sadeek and Rocco Racioppi; Investigation, Ippolito Camele, Sadeek Sadeek and Hazem Elshafie; Methodology, Sadeek Sadeek, Rocco Racioppi and Hazem Elshafie; Supervision, Ippolito Camele and Sadeek Sadeek; Writing – original draft, Hazem Elshafie; Writing – review & editing, Ippolito Camele and Hazem Elshafie.

Funding: This study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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